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Oligonucleotide-selenide conjugate: Synthesis and its inducible sequence-specific alkylation of DNA

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ABSTRACT

Oligonucleotide-selenium conjugate was designed and synthesized and its sequence-specific cross-linking ability was investigated. The selenide derivatives can generate covalent interstrand cross-linking with its complementary strand through the formation of *o*-QM intermediate induced by periodate oxidation. A cross-linking reaction yield of up to 50% was obtained. Hydroxyl radical footprinting experiment revealed that the quinone appendage specifically alkylated the cytosine base extending the duplex formed between the conjugate and the target strand.

1. Introduction

Sequence-selective interstrand cross-linking (ISC) between complementary duplexes or triplexes[1–3] is a topic of intense research interest, due to its potential applications in basic research and clinical use. This strategy is expected to inhibit transcription and expression of specific genes using antisense strategies, and it is developing as a tool for site-directed chemical modification that may induce point mutations in the genetic code.[4]

The DNA cross-linking reaction is the primary mechanism for the cytotoxic activity of many clinical antitumor drugs, such as nitrogen mustards, platinum agents and mitomycin C.[5] The basic principle of cross-linking includes the incorporation of a reactive functional group into the DNA by covalent bonding. This non-reversible covalent bond formation between two DNA strands can disrupt the maintenance and replication of tumor cells DNA and eventually lead to cell death.[6] Unfortunately, many existing alkylating and cross-linking agents have functional groups with high intrinsic reactivity and of considerable instability. These agents are also susceptible to nucleophilic attack by a number of naturally-occurring chemicals such as water, amino and sulfidryl groups. In addition, these agents, even those already used clinically as antitumor drugs, are poorly selective and highly toxic. Therefore, in recent years, attention has shifted towards improving the target selectivity of cross-linking agents.

The ideal solution to such problems is the use of drugs that can be brought selectively close to the target, and whose cross-linking activity can be induced by a trigger, such as photo-irradiation, enzymatic or chemical stimulus.

The inducible formation of *o*-quinone methide (*o*-QM) as a precursor of antitumor agents has been reported by many groups because *o*-QM motif is highly reactive with DNA.[7] Our group has reported several efficient DNA ISC agents derived from an *o*-QM intermediate induced by a biphenol quaternary ammonium structure,[8] a biphenol selenide structure[9] and a bis (catechol) quaternary ammonium structure.[10] In this communication we now report the successful production of a sequence-selective alkylating agent that can be induced with high efficiency under mild oxidative conditions.

2. Results and discussion

2.1. Chemistry

3-(4-Hydroxyphenyl) propionic acids **1** (Scheme 1),[11] subjected to successive hydroxyl group protection, bromination, substitution and deprotection, yielded the acid derivative **2**. Treatment of the acid **2** with *N*-hydroxysuccinimide in the presence of

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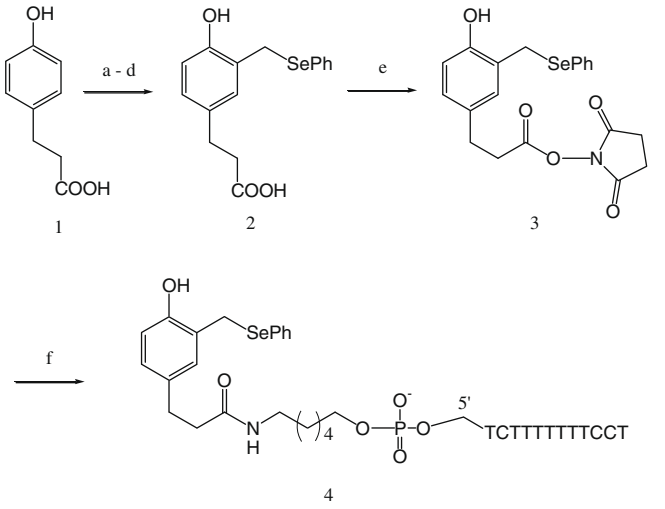


Scheme 1. Synthesis of compounds **1**–**4**. Reagents and conditions: (a) β-(4-hydroxyphenyl) propionic acid, K_2CO_3, $(CH_3)_2SO_4$, acetone, rt, 95%; (b) $(CH_2O)_n$, HBr, CH_3COOH, 61%; (c) $(PhSe)_2$, Zn, NaH_2PO_4, H_2O, CH_3CN, rt, 90%; (d) BBr_3, CH_2Cl_2, 45%; (e) AcOEt, NHS, DCC; (f) $NaCO_3/NaHCO_3$ buffer, pH 7.5.

N,*N*′-dicyclohexylcarbodiimide yielded the activated ester **3**. This in turn was used for coupling to oligonucleotides modified at the 5′ terminus with a hexamethyleneamino linker. The final product **4** was then purified by reverse-phase chromatography and confirmed by UV and MALDI-TOF mass spectrometry.[11] A target DNA strand, 5′-d(GAACTCTAGGAAAAAAAGACTAAG) **5**, was also prepared and labeled at the 5′ terminus with TAMRA (TAMRA = 6-carboxytetramethylrhodamine, succinimidyl ester). The 5′ amino linker derivative and other oligonucleotides were synthesized using the standard procedures of solid-phase phosphoramidite chemistry.

2.2. DNA cross-link

Oligonucleotide derivative **4** was first annealed to its complementary strand **5** and then treated with multiple concentrations of sodium periodate ($NaIO_4$), a mild oxidative agent that does not react with proteins and nucleotides,[11] prior to analysis by 20% denaturing polyacrylamide gel electrophoresis. Treatment of the annealed duplex with sodium periodate led to the formation of a slowly migrating band in the gel. The electrophoresis velocity of the new high molecular weight products was similar to that of a 39-mer oligonucleotide used as a molecular weight marker, indicating the formation of cross-linked products consistent with the covalent attachment of the complementary duplex (Fig. 1).

The oxidation-dependent activation described above is consistent with previously reported mechanisms.[12] This target-selective ISC reaction proceeded via an electrophilic intermediate as *o*-QM formed under oxidative conditions (Scheme 2). No high molecular weight species were observed in the absence of oxidant (Lane 1). In addition, the transient intermediate generated upon oxidation could be trapped by ethyl vinyl ether (EVE), a trapping agent for *o*-QM, to produce the expected QM-EVE adducts.[9] The strategy of using a non-reactive precursor **4** can prevent the side effects caused by the intrinsic high reactivity of functional groups in some other alkylating agents. The most effective point is that the cross-linking reaction can only take place when specifically induced after suitable binding with the target. After hybridization of **4** and **5**, the ISC was dependent on the concentration of $NaIO_4$ added to the medium. Reaction yields of up to 50% (Lane 6, determined by densitometry) were obtained at a moderate oxidant concentration (20 mM). This is consistent with our previously published results

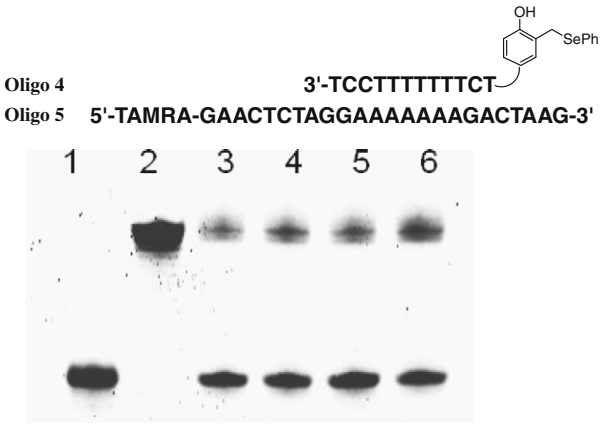


Figure 1. Denatured PAGE (20%) was used to identify the concentration dependence DNA cross-linking ($NaIO_4$ oxidation). Oligonucleotides **4** and **5** (2 μM in each strand) were annealed in 20 mM PBS, pH 7.4, 30 °C, and treated with the following concentrations of $NaIO_4$. Lane 1: none; Lane 3: 1 mM $NaIO_4$; Lane 4: 5 mM $NaIO_4$; Lane 5: 10 mM $NaIO_4$; Lane 6: 20 mM $NaIO_4$; Lane2: 39-mer oligonucleotide used as a molecular weight marker.

that phenyl selenide derivatives were capable of cross-linking DNA through the formation of *o*-QM.[9]

In control experiment, no cross-link product could be observed for the partially-complementary oligonucleotide **6 5′-d(GAACTCT AGGTAAAAATTACTAAG),** (Fig. 2).

2.3. Determination of the alkylation site

To identify the specific reactive sites of the complex, the cross-linked product was purified by gel electrophoresis and subjected to a highly effective method of footprinting based on Hopkins' hydroxyl radical cleavage of DNA.[13] Interstrand modification is revealed by the contrast in scission fragmentation from the native strand and the cross-linked DNA. The first base extended from the duplex, C20, was alkylated by the quinone appendage (Fig. 3). Selective alkylation of cytosine is consistent with previous reports showing that a quinone methide intermediate modifies the N3 position of cytosine[14] with a more than 10-fold preference over purines.[15]

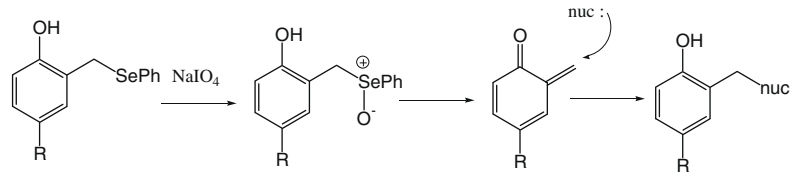
3. Conclusion

In conclusion, we have synthesized oligonucleotide-selenium conjugate and have demonstrated its capability to perform highly efficient and selective DNA cross-linking reactions without incurring chemical instability. The selenide derivatives can perform ISC through the formation of *o*-QM induced by periodate oxidation. Future efforts will focus on the biological applications of the new cross-linking motifs in antisense strategies or site-directed chemical modification of cytosine.

4. Experimental section

4.1. 3-(4-Methoxyphenyl)propionic acid methyl ester, 1a[16]

Commercially available 3-(4-hydroxyphenyl)propionic acids (1.68 g, 10 mmol) was dissolved in 40 mL acetone and methyl sulfate (5 mL). Then solid carbonate potassium (6.90 g, 50 mmol) was added and the mixture was refluxed for 48 h. The reaction mixture was filtered through a short pad of silica gel, and the filtrate was concentrated to afford 3-(4-methoxyphenyl)propionic acid methyl ester (**1a**) (1.84 g, 95% yield) as a colorless liquid. ^{1}H NMR ($CDCl_3$) δ 2.60 (t, J = 8.0 Hz, 2H), 2.89 (t, J = 8.0 Hz, 2H), 3.66 (s, 3H), 3.78 (s, 3H), 6.83 (d, J = 8.6 Hz, 2H), 7.13 (d, J = 8.6 Hz, 2H).



Scheme 2. Proposed mechanism for interstrand cross-link (ISC) formation.

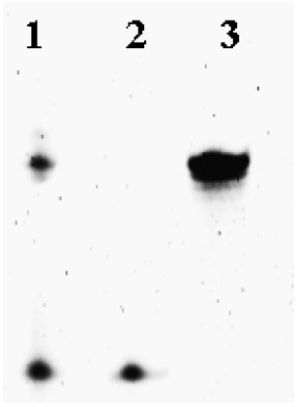


Figure 2. Denatured PAGE (20%) was used to identify the concentration dependence DNA cross-linking ($NaIO_4$ oxidation). Lane 1: oligonucleotides **4** and **5** (2 μM in each strand), Lane 2: oligonucleotides **4** and partially-complementary oligonucleotide **6** (2 μM in each strand) were annealed in 20 mM PBS, pH 7.4, 30 °C, and treated with 20 mmol $NaIO_4$; Lane 3: 39-mer oligonucleotide served as a molecular weight marker.

4.2. 3-(4-Methoxy-3-bromethylphenyl)-propionic acid methyl ester, 1b[17]

A solution consisting of 12 mL glacial acetic acid, **1a** (1.94 g, 10 mmol) and paraformaldehyde (0.45 g, 15 mmol) was heated at 70 °C for 15 min. Then 3.5 mL 33% HBr in acetic acid was added dropwise during 5 min. After 20 min at this temperature, the mixture was cooled to 0 °C for 12 h. The reaction mixture was then poured into cold water, and extracted with ethyl acetate three times. All organic phase was combined and washed with saturated aqueous $NaHCO_3$, and concentrated in vacuo. The residue was purified by column chromatography (cyclohexane/ethyl acetate = 4:1) to yield the desired product as a white solid (1.74 g, 61% yield). ^{1}H NMR ($CDCl_3$) δ 2.60 (t, J = 8.0 Hz, 2H), 2.88 (t, J = 8.0 Hz, 2H), 3.67 (s, 3H), 3.87 (s, 3H), 4.53 (s, 2H), 6.80 (d, J = 5.1 Hz, 1H), 7.14 (t, J = 7.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 29.27, 30.16, 36.03, 51.83, 55.90, 111.33, 126.28, 130.13, 131.02, 132.90, 156.22, 173.47. HRMS (ESI) m/z, calcd for $C_{12}H_{15}O_3$ $(M-Br)^+$ calcd 207.1016. Found 207.1018.

4.3. 3-(4-Methoxy-3-phenylselanylmethyl-phenyl)-propionic acid methyl ester, 1c[18]

To a solution of NaH_2PO_4 (6.0 g, 43.5 mmol) in 6.0 mL of water, **1b** (0.58 g, 2 mmol) and $(PhSe)_2$ (0.473 g, 1.5 mmol) dissolved in 6 mL CH_3CN were added. Under vigorous stirring, Zn powder (0.26 g, 4 mmol) was added. After stirring for 1.5 h at room temperature, the mixture was hydrolyzed and extracted with ethyl acetate. The organic phase was evaporated to dryness and the solid was purified via silica gel column chromatography (cyclohexane/ethyl acetate = 8:1) to give product **1c** as a yellow oil (655 mg, 90% yield). ^{1}H NMR ($CDCl_3$) δ 2.49 (t, J = 8.0 Hz, 2H), 2.78 (t, J = 8.0 Hz, 2H), 3.66 (s, 3H), 3.78 (s, 3H), 4.08 (s, 2H), 6.76 (m, 2H), 7.01 (d, J = 7.8 Hz, 1H), 7.24 (m, 3H), 7.45 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 27.17, 30.23, 36.16, 51.82, 55.74, 110.86, 127.44, 127.56, 128.10, 129.02, 129.07, 130.28, 132.34, 134.23, 134.28, 134.34. HRMS (ESI) m/z, calcd for $C_{18}H_{20}NaO_3Se$ $(M+Na)^+$ calcd 387.0470. Found 387.0468.

4.4. 3-(4-Hydroxy-3-phenylselanylmethyl-phenyl)-propionic acid (2)

Under nitrogen atmosphere, at 0 °C, BBr_3 (neat, 0.26 mL, 2.88 mmol) was added gradually to a solution of **1c** (182 mg, 0.5 mmol) in freshly distilled CH_2Cl_2 (10 mL). After the mixture was stirred for 2 h at room temperature, the solution was poured into a large amount of ice-water and extracted with ethyl acetate. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography (dichloromethane/ethyl ether = 1:1) to give the desired product as a yellow oil (76 mg, 45% yield). ^{1}H NMR (CD_3OD) δ 2.39 (t, J = 8.0 Hz, 2H), 2.68 (t, J = 8.0 Hz, 2H), 4.08 (s, 2H), 6.68 (m, 2H), 6.87 (m, 1H), 7.22 (m, 3H), 7.45 (m, 2H); HRMS (ESI) m/z, calcd for $C_{16}H_{16}NaO_3Se$ $(M+Na)^+$ calcd 359.0157. Found 359.0153.

4.5. Conjugate 4[19]

To an ice-cold solution of 30 mL ethyl acetate including acid **2** (108 mg, 10 mM) and *N*-hydroxysuccinimide (NHS) (34 mg, 10 mM), a solution of *N,N′*-dicyclohexylcarbodimide (DCC) (62 mg, 10 mM) in 15 mL ethyl acetate was added dropwise. After stirring for 16 h, the mixture was allowed to warm up to room temperature. The precipitate was filtered off, and filtrate was evaporated in vacuum to yield the activated NHS ester. The ester was directly used for the conjugation reaction without further purification.

The appropriate oligonucleotide containing a 5′-hexamethyleneamino linker (80 nmol) in $NaHCO_3/Na_2CO_3$ buffer (100 mM, pH 7.5, 30 μL) was mixed with an equal volume of NHS ester in DMF and incubated at ambient temperature for 12 h. The desired conjugate **4** was then isolated after purification by reverse-phase chromatography with a C-18 column and a gradient of 5–50% acetonitrile in an aqueous solution of triethylammonium acetate (50 mM, pH 7) over 25 min (1 mL/min). ESI-MS, calcd 4041.4, found 4041.69.

4.6. Cross-linking reaction of oligonucleotides 4 and 5

Cross-linking reaction was performed with oligonucleotides **4** and **5** annealed in PBS buffer (9 μL, 20 mM, pH 7.4) for 10 min at 30 °C. Then $NaIO_4$ in PBS solution was added at the final volume of 10 μL and a final concentration of 2 μM duplex. The mixture was incubated at 30 °C and the reaction was quenched after 24 h with the addition of 10 μL formamide deionized. The reaction products were then analyzed on a 20% denaturing polyacrylamide gel electrophoresis.

4.7. Hydroxyl radical cleavage of DNA by Fe(II)·EDTA[20]

The cross-linked products were isolated by polyacrylamide gel electrophoresis.[1] The Fe(II)·EDTA cleavage reactions were conducted as previously described by Hopkins.[2] Cross-linked DNA

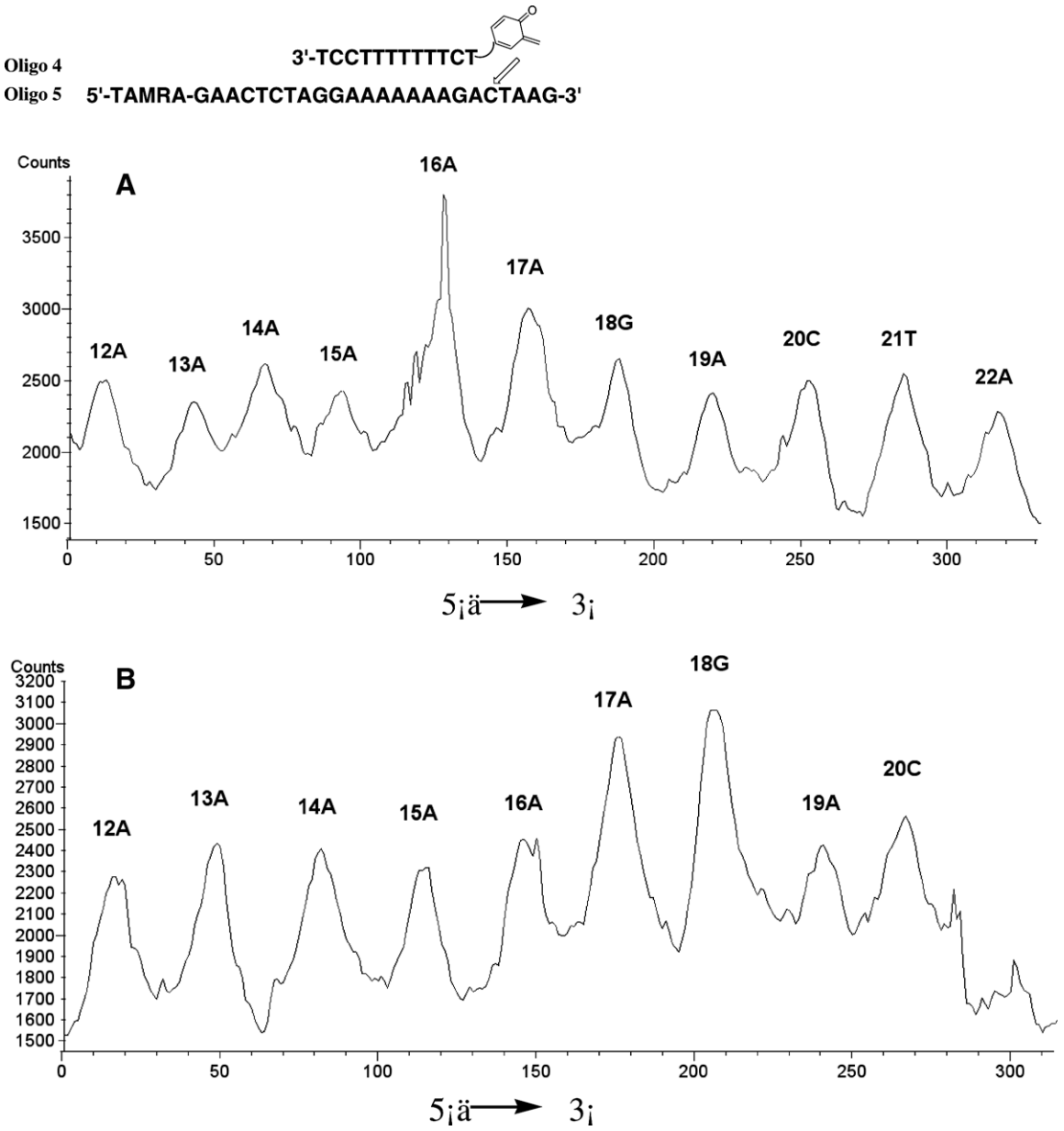


Figure 3. Densitomeric scans representing hydroxyl radical-generated footprints of hybridized and cross-linked DNA. (A) Fragmentation of a control sample. (B) Fragmentation of the alkylated product.

(10 pmol) was treated with a solution of 500 μM $(NH_4)_2Fe(SO_4)_2$, 1 mM EDTA, 10 mM sodium ascorbate, 0.1 M H_2O_2, 10 mM phosphate buffer (pH 7.7) in a total volume of 10 μL for 1 min at room temperature, and quenched by the addition of 1 μL of 0.1 M thiourea. Samples were lyophilized and analyzed by denaturing PAGE.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.026.

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